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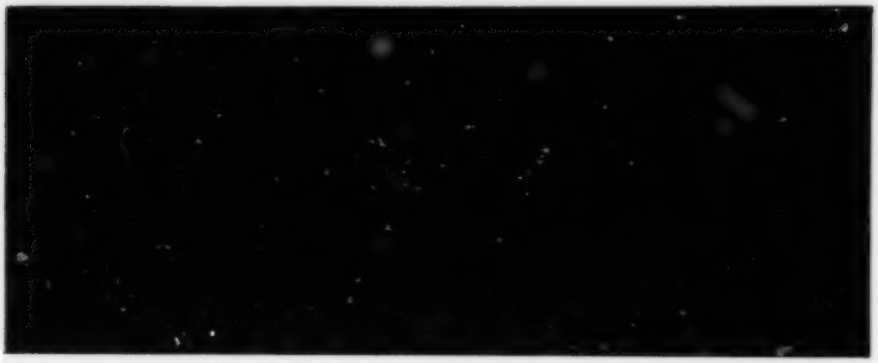

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Final Report

E-86-01-0007

Investigating Chloride Conductance Inhibitors as Treatments for Neonatal and Weanling Diarrhea in Pigs

Conducted by Dr. G. Forsyth

Funded by the Agriculture Development Fund

Final Report for the ADF project E-86-01-0007, Investigating Chloride Conductance Inhibitors as Treatments for Neonatal and Weanling Diarrhea in Pigs.

a) Executive Summary

Abstract

The major objective of the research project was to investigate the properties of the ion transport protein in intestinal cell membranes which allows the electrolyte and fluid losses responsible for the clinical symptoms of neonatal colibacillosis and weanling diarrhea in pigs. We proposed a study of the ion transport properties of the protein, of how the ion transport capacity increases in response to bacterial enterotoxins, of the effects of recently discovered ion transport inhibitors on the transport capacity of the protein, and of the efficacy of these ion transport inhibitors as treatments for diarrheal disease in pigs. The basic conclusions from the project indicate that we can use cell membrane preparations isolated from pig intestine to show activation of ion transport, and blockage by inhibitors, but the same inhibitors are not effective blockers of secretory ion transport when put into the intestine or the blood of live pigs.

Summary

Results

The ion transport properties of the chloride conductance protein were characterized early in the study, using membrane vesicles prepared from scrapings of the cells lining the pig small intestine. This ion transport was shown to have the properties of the transport process which is responsible for the fluid and electrolyte losses in diarrheal disease caused by enteropathogenic bacteria. These findings indicated that the model system using membrane vesicles should be a valid approach for studying intestinal ion transport without the need to use live animals on a daily basis.

Activation of chloride ion transport by this membrane protein is a key feature of diarrheal disease. We expended a lot of effort to establish the conditions for activating chloride ion transport in the intestinal membrane vesicle system. These studies were successful where many others have failed because we were lucky enough to employ a buffering agent in the vesicle preparations which prevented spontaneous activation found by other investigators of the conductive chloride transport. There are important implications for a system which activates spontaneously in response to changes in the solution buffering. For example, it is possible that spontaneous activation represents the loss or the removal of an inhibitory component of the chloride transport protein. Other investigators working with submicroscopic pieces of cell membrane and sophisticated electrical apparatus have recently reached similar conclusions. Our system has the advantage that we are using large amounts of intestinal cell membrane protein, and we are presently devising ways to study the structure and function of the inhibitory component which limits chloride ion release into the gut lumen, thereby preventing diarrhea. More detailed knowledge of this inhibitory component should eventually lead to better methods of direct interference with diarrheal disease.

The inherited human condition, cystic fibrosis, is apparently due to a genetic defect in the inhibitory component of the chloride conductance channel which prevents normal fluid secretion into the intestine and the lungs of affected individuals. Our research findings are also of interest in relationship to this human disease. The connection between too much secretory activity in diarrheal disease, and too little secretory activity in cystic fibrosis makes the study of the inhibitory component of the chloride ion channel an important area of current research.

Most of the recognized inhibitors of chloride transport are known to be relatively nonspecific. This means that these inhibitors will block chloride transport processes other than the one involved in ion and fluid secretion in diarrheal disease. There are many essential chloride transport pathways in several body tissues which cannot be altered without causing significant problems to the general health of an animal. We have synthesized our own supply of a newly reported chloride transport inhibitor as well as screening several commercially available organic acids in hopes of finding a highly specific inhibitor which would be well suited for experimental trials as an antidiarrheal.

As a result of these experiments we have found that α-phenylcinnamic acid appears to be much more specific for the secretory chloride transport pathway than existing recognized chloride transport inhibitors. Our finding was greeted with considerable interest at the March 1989 meeting of the Federation of American Societies for Experimental Biology. We are currently using α-phenylcinnamate as a specific label in trying to identify and to isolate the chloride conductance protein from pig intestinal mucosal cells.

The final part of our experiments for the project has been the testing of chloride transport inhibitors as inhibitors of intestinal secretion in weanling pigs. We have measured both fluid and chloride secretion into ligated intestinal loops prepared surgically in pig jejunum and ileum. This part of the project has been a real disappointment because these compounds which work very well to block chloride transport in membrane vesicles prepared from pig intestine had no detectable effect on chloride transport in living cells lining the pig gut. We have tried several inhibitors, including α-phenylcinnamate, two secretory agents, two different locations in the small intestine, and three different routes of administration without finding significant reductions in chloride or fluid secretion. It seems likely that the glycocalyx lining of the gut may act as a permeability barrier to prevent chloride transport inhibitors from reaching the membrane transport protein. We have used a morphine-like compound called loperamide as a positive control to verify that secretory inhibition is possible in our system. Loperamide was able to reduce both fluid and chloride secretion into surgically prepared intestinal loops, and it is possible that this compound which has been used extensively in humans may also hold some promise for use as an antidiarrheal in pigs.

Impact on Saskatchewan Agriculture

The lack of antidiarrheal effects of chloride transport inhibitors is a real disappointment in this study, and the report has been written to give a realistic assessment of the current knowledge in this area. Perhaps it is also appropriate to look at the longer term, and to realize that additions to the basic knowledge about intestinal fluid and electrolyte transport will one day be used to achieve the goals of this research project - to provide quick and effective suppression of the harmful effects of diarrheal disease without direct and immediate concerns about killing the bacterial pathogens responsible for the problem. In this respect we can be more positive about the project results because significant new information has been generated, and the benefits of this information and subsequent findings will eventually be available to all Saskatchewan livestock producers in terms of increased ease of treatment and reduced neonatal mortality from bacterial diarrheal disease.

b) Technical Report

As the suggested headings for the technical report section are identical to those used in scientific journals, and as the project has involved several discrete experimental studies, it seems appropriate to present the technical report as the series of manuscripts prepared to describe the results of the research. Please see attachments for the details of this technical information.

c) Papers Prepared and Talks Given

Manuscripts

Forsyth G.W. & S.E. Gabriel. Chloride ion transport into pig jejunal brush-border membrane vesicles. J. Physiol. **402**:555-564, 1988.

Forsyth G.W. & S.E. Gabriel. Activation of chloride conductance in pig jejunal brush border vesicles. J. Membrane Biol. **107**:137-144, 1989.

Forsyth G.W. & S.E. Gabriel. α-Phenylcinnamate inhibits chloride conductance in jejunal brush border membrane vesicles. Biochim. Biophys. Acta in press (accepted June 22, 1989).

Forsyth G.W. & S.E. Gabriel. Chloride conductance inhibitors do not block ileal secretion caused by cholera toxin or theophylline. submitted Am. J. Vet. Res. July 7 1989.

c) continued

Oral presentations

Forsyth G.W. Activation of Chloride Conductance in brush border membrane vesicles. invited presentation at the Banff Biomembranes Symposium, May 1987.

Forsyth G.W. Chloride conductance in cystic fibrosis. U of Saskatchewan, Department of Biochemistry Seminar Series, Jan 25, 1989.

Poster presentations:

Gabriel S.E. & G.W. Forsyth. Reconstitution of chloride conductance activity of the avian salt gland. Banff Biomembranes Symposium, May 1987.

Gabriel S.E. & G.W. Forsyth. Fluorescein labelling of the chloride conductance protein. Federation of the American Societies for Experimental Biology, March 1989.

d) **Verified Statement of Expenditures**

The information for this statement is expected to be available from the University Research Accounts Office at the end of August, 1989.

e) **Personnel and Salaries**

Genia Olender has been employed continuously as a technical assistant for the duration of the project, with the exception of four months of maternity leave in the summer of 1988. Her full salary has been charged to the project. $54643.17

Lucy Kumar was employed as a maternity leave replacement for Genia in the summer of 1988. Her salary was also charged entirely to the project. $10004.46

Sherif Gabriel has been supported for a ten month period each calendar year for the duration of the project. There was an increase in rate of pay for the other two months so the ADF support amounted to about 80% of his annual salary. $22174.87

Darryl Caves worked as a summer student in 1987. Fifty percent of his salary came from ADF sources. $2991.18

Eva Yonge was also a technical assistant. She was supported by MRC funds, but received a small amount of support (less than 1%) from ADF in 1987. $173.88

Total Salary Expenditures for all personnel employed on the project. $89,988.18

f) **Equipment Purchased**

The only equipment item obtained from project funding with a value greater than $500 was a vacuum filtration manifold for use in measuring chloride uptake by vesicles. This was purchased from Technical Marketing Associates at a cost of $1183.85 in October of 1986.

Dan Forsyth

July 28, 1989

Chloride conductance inhibitors do not block ileal secretion caused by cholera toxin or theophylline

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Compounds which prevent chloride transport in membrane vesicles have been tested for in vivo activity against the effects of intestinal secretory agents. Chloride channel blockers including diphenylamine-2-carboxylate, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate, 5-nitro-2-(2-phenylethylamino)benzoic acid and α-phenylcinnamic acid were tested for effects on jejunal or ileal secretion in weanling pigs. Secretion was studied in ligated intestinal loops in a control state, during exposure to secretory levels of theophylline, and after prior treatment with cholera toxin. Increases in net fluid flux induced by either theophylline or cholera toxin were not prevented by adding chloride channel blockers into the gut lumen. Channel blocker concentrations which reduced chloride transport by >50% in pig jejunal brush border vesicles did not cause significant changes in unidirectional blood to lumen chloride flux, $J^{Cl}_{s>m,}$ measured in situ. Several routes of administration of the specific chloride channel blocker α-phenylcinnamate failed to reduce fluid secretion induced by theophylline. There appears to be a significant difference in chloride channel blocker effectiveness between in vitro and in vivo experimental models.

In contrast to the chloride channel blockers, loperamide significantly reduced net fluid and chloride flux in ileal loops secreting fluid in response to theophylline. Antagonism of the production or actions of second messenger by loperamide was more effective than the chloride channel blockers in reducing conductive chloride transport associated with intestinal secretion.

Introduction

Conductive chloride secretion is assumed to regulate the movement of fluids from the blood to the intestinal lumen. This process has an important role in maintaining the fluidity of the intestinal contents to aid in intestinal transit, and nutrient digestion and absorption. Excessive chloride secretion via hyperactivity of this channel is assumed to be the cause of secretory diarrheal disease arising from intestinal colonization by enteropathogenic bacteria. Bacterial enterotoxins act via binding to surface receptors, transduction of the binding signal to intracellular second messenger production, activation of protein kinases by second messengers, and subsequent alteration of ion channel transport activity via phosphorylation by activated protein kinases. The complexity of the secretory mechanism gives a choice of sites for interfering with intestinal secretion by appropriate pharmacologic agents.

Considerable effort has been directed toward controlling second messenger production, release or activity[1]. Zenser reported that adenosine analogues would inhibit adenylate cyclase activity in intestinal epithelial cells[2], but this approach was not effective in reducing intestinal secretion in an experimental model[3]. Effective antisecretory agents including the phenothiazines[4], β-blockers[5], enkephalin agonists[6,7] and somatostatin[8] may be acting via interference with calcium-dependent processes and protein kinase activity.

More recently the chloride channel blockers have been emphasized as experimental tools for studying mechanisms in secretory diarrhea and as possible pharmacologic agents to treat acute hypersecretion[9]. These agents have the obvious advantage of working directly at the site of ion secretion into the gut lumen, and avoiding the undesireable side effects of drugs which modify second messenger

activity. Chloride channel blocker activity has been studied at the level of single channels[10,11], membrane vesicles[9,12], and intact mucosal sheets mounted in Ussing chambers[13]. The channel blockers appear to be promising compounds for blocking chloride secretion in each of these models, so it seems appropriate to test them in an in vivo intestinal system. This study was carried out to compare the effects of several reported chloride channel inhibitors on the intestinal secretory response of the weanling pig to theophylline and to cholera toxin.

Materials and Methods

All measurements of fluid and chloride flux were carried out in weanling pigs of mixed Yorkshire-Landrace breeding. Weanling pigs weighing 15 to 20 kg were induced and maintained on halothane anaesthesia for the course of flux measurements. Up to 12 ligated intestinal loops 15 cm in length were prepared in the jejunum (starting 15 cm distal to the ligament of Treitz), or in the ileum (proceeding proximally from the beginning of the ileocecal ligament). Care was taken to avoid any interference with the blood supply during loop preparation. Loops were fitted with lucite cannulae, and carefully returned to the peritoneal cavity except for brief periods during addition and withdrawal of solutions.

Jejunal flux studies were carried out in anterior loop fluid (Na, 140; K, 5; Cl, 125; and HCO_3, 20 mM, containing polyethyleneglycol-2000 (PEG) 1.0 g/l, 2.0 µCi ^{3}H PEG)[14]. For ileal flux studies the loop fluid was modified by changing the Cl and HCO_3 to 40 and 105 mM, respectively. ^{36}Cl (1 µCi/l) was added to the loop fluids for unidirectional Cl flux measurements. Secretory effects of theophylline were measured by adding 15 mM theophylline to the loop fluid, while control loop fluid contained 15 mM mannitol. Flux studies with theophylline were carried out

for 20 min as described by Hamilton et al.[14].

Cholera toxin was used in the form of a lyophilized broth filtrate (150 mg / loop) which has a secretory potency equivalent to a cholera toxin content of 0.1% on a weight basis[15]. Cholera toxin was added to loops in 10 ml of isotonic saline, and incubated in the loops for 90 min prior to removal for flux measurements. Flux was measured over the 20 min period following cholera toxin removal using anterior or distal loop fluid as described above. Volume changes were calculated from changes in concentration of ^{3}H PEG as reported previously[14]. Unidirectional Cl fluxes were calculated using the kinetic model of Berger and Steele[16].

Chloride concentrations in loop contents at the end of the flux period were determined by coulometric-amperometric titration. ^{3}H and ^{36}Cl were measured using a dual label program in a Beckman LS-3800 liquid scintillation counter.

Diphenylamine-2-carboxylate (DPC), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) and cis-α-phenylcinnamic acid (α-PC) were purchased from commercial suppliers. 5-Nitro-2-(2-phenylethylamino)benzoic acid (NPEB) was synthesized according to Wangemann et al.[17], and the product was purified by thin layer chromatography and C:18 reverse phase HPLC. Elemental analysis and nuclear magnetic resonance spectroscopy were used to confirm the identity of the NPEB (analysis by the Department of Chemistry, University of Saskatchewan).

Intraluminal effects of chloride channel blockers were determined by including the blockers in the anterior or in the distal loop fluid during the course of the fluid flux. The choice of blocker concentrations for testing was based on levels shown to be effective in in vitro trials of Cl uptake by jejunal brush border vesicles [12,18] For studies with cholera toxin as secretogogue the inhibitors were allowed to preincubate

in the loops for five minutes and maintained in the loops for the duration of the 20 minute flux period. For intralumenal testing with theophylline as secretogogue the inhibitors were present with the theophylline, from the onset of the secretory stimulus. When intraperitoneal and intravenous routes of administration were used the blockers were given 5 min prior to initiating flux measurements.

Procedures for brush border membrane vesicle preparation, and chloride flux measurements across ileal brush border vesicle membranes were performed as reported previously[12,19]. Significant treatment effects were determined by comparison to controls, using Student's T-test.

Results

An effective chloride channel blocker should have specificity for the conductive chloride channel, as well as a reasonably high affinity, or ability to displace chloride from a transport site. α-PC was tested in the in vivo model for secretory diarrhea because it was shown to have more selectivity for conductive chloride secretion than SITS, DPC, or NPEB[18]. In addition α-PC is an uncompetitive inhibitor of chloride uptake by pig intestinal brush border vesicles, indicating that it should not be displaced from the conductance channel by the chloride concentrations encountered in the intestinal lumen[18].

Incubation of cholera toxin in pig jejunal loops for a period of 90 minutes or longer produced a sustained secretory response which persisted after removal of the enterotoxin from the intestinal loops[15]. The effects of the α-PC on net fluid flux in the jejunal loops is shown in Table 1. Concentrations of α-PC which were quite effective as inhibitors of Cl conductance in jejunal brush border vesicles did not reduce the secretory net fluid flux response to cholera toxin. The established Cl

conductance inhibitors DPC and NPEB were also without effect on the net secretory fluid flux induced by cholera toxin.

Jejunal fluid secretion is a normal physiological state in the pig, although it may decrease in magnitude with dehydration and the aboral distance from the ligament of Treitz[14,15]. Bicarbonate secretion may be equally important as chloride conductance as a driving force for this net secretion[14]. The distal ileum of the pig is normally in an absorptive state, and may be more typical of the classical model for intestinal fluid secretion driven by increased chloride conductance. The effect of a range of concentrations of conductance inhibitors in the intestinal lumen on net fluid flux in ileal loops is shown in Table 2. DPC was the only inhibitor which showed a trend toward increased absorptive fluid flux at higher concentrations, although the flux value at $1 \cdot 10^{-2}$ mol l^{-1} was not significantly higher than the control flux ($P = 0.083$). The other inhibitors actually tended to decrease the absorptive flux, although the decreases were also not statistically significant.

Ileal fluid secretion in vivo is accompanied by significant increases in unidirectional serosal to mucosal chloride flux ($J^{Cl}_{s \to m}$)[14]. Theophylline causes secretory responses in the pig jejunum or ileum which are quantitatively and qualitatively indistinguishable from those produced by cholera toxin. Theophylline was used in the next series of experiments to allow for flux measurements to be made in the same ileal loops before and after the addition of α-PC. Unlike cholera toxin, the secretory effect of theophylline is readily reversible on removal of the agent from the intestinal lumen. For these experiments theophylline was added to the distal loop fluid (15 mmol$\cdot l^{-1}$), and was present in the lumen of the loops during the 20 minute interval of flux measurements. Addition of α-PC (2.0 mmol$\cdot l^{-1}$) to the

distal loop fluid did not reduce the net secretory response to theophylline or cause a significant reduction in $J^{Cl}_{s\to m}$ (Table 3).

Alternate routes of administering α-PC were tested to determine if the lack of effect on chloride conductance, as measured by $J^{Cl}_{s\to m}$, was due to poor delivery via the intestinal lumen. For both intraperitoneal and intravenous administration of α-PC the dose was calculated to provide a concentration of 2.0 mmol l^{-1} in the extracellular fluid. Net ileal fluid secretion induced by theophylline was not reduced when α-PC was supplied in the intestinal lumen or via the intra-peritoneal route. Only for the intravenous route was there a trend (P = 0.27) toward reduced net fluid secretion with α-PC. There was also a trend toward reduced $J^{Cl}_{s\to m}$ with both intraperitoneal (P = 0.10) and intravenous (P = 0.11) routes of α-PC treatment. The biological significance of the trend is not clear since net fluid secretion was not significantly reduced.

α-PC shows uncompetitive inhibition of chloride uptake by jejunal brush border vesicles, with a K_i value in vitro of approximately 0.5 to 1.5 mmol l^{-1} [18]. In comparison to 2.0 mmol l^{-1} concentrations of α-PC used in these trials it could be suggested that the affinity of the putative inhibitor for the chloride conductance channel might limit inhibitor effectiveness in vivo. Chloride conductance inhibitors with much higher affinities for the channel have been reported. NPEB and its propyl analogue are reported to have affinities in the 10^{-7} molar range[17]. Thus they might be predicted to show inhibition of in vivo $J^{Cl}_{s\to m}$ even if non-specific effects on other anion transport proteins could limit secretory inhibition as measured by net fluid flux. This hypothesis was tested in ileal loops treated with either theophylline or cholera toxin and the results for secretion induced by cholera toxin are shown

in Table 4. NPEB at 2.0 mmol l^{-1} did not reduce either net fluid flux or $J^{Cl}_{s \to m}$ in this trial. Employing lower concentrations of NPEB, or testing NPEB against secretory effects of theophylline also gave no evidence for inhibition of ileal secretion by this compound (data not shown).

Loperamide is an example of an antisecretory agent which is used extensively in human medicine, but has only received limited testing for antisecretory effects in domestic animals[20]. Ahsan et al. have reported that loperamide may affect unidirectional chloride flux in rabbit ileum mounted in Ussing chambers[21]. Loperamide was chosen to try to obtain a positive control in the pig for comparison with the chloride channel blockers. The effects of loperamide on the net secretory fluid flux induced by exposure of ileal loops to intraluminal theophylline is shown in Fig 1. The concentrations of loperamide used in the trial did not affect the net fluid flux in control loops (no theophylline added). Net fluid secretion caused by theophylline was reduced by loperamide in a dose-related fashion. The antisecretory effect of loperamide was not complete (control, -13±10 µl per loop per minute vs theophylline & 100 µmol·l^{-1} loperamide, 24±7 µl per loop per minute; P = 0.007) and higher doses of loperamide did not give additional antisecretory activity (data not shown). Antisecretory effects of loperamide occurred via a trend to reductions in $J^{Cl}_{s \to m}$ (P = 0.181 for comparing zero vs 100 µmol·l^{-1} loperamide per ml distal loop fluid)(Fig 2). A similar trend was observed toward increased $J^{Cl}_{m \to s}$ with loperamide treatment (P = 0.30). As a result of these two converging trends toward reduced unidirectional secretion and increased unidirectional absorption, the net chloride flux after theophylline treatment, J^{Cl}_{net}, was significantly reduced at both 20 (P = 0.013) and 100 µmol·l^{-1} (P = 0.012) loperamide.

The seeretory responses to theophylline and cholera toxin are usually considered to be indistinguishable in terms of all steps subsequent to accumulation of intracellular cAMP. Concentrations of loperamide which blocked the secretory effects of theophylline did not reduce the increase in net fluid flux caused by exposure to cholera toxin (Table 5). J^{HOH}_{net} was 15±6 µl per loop per minute after treatment with cholera toxin, and 15±8 when 100 µmol·l^{-1} loperamide per ml of distal loop fluid was present during the flux measurement. In spite of the lack of effect of loperamide on J^{HOH}_{net}, there was a trend to reduced $J^{Cl}_{s->m}$ with loperamide. Higher concentrations of loperamide up to 2 mmol·l^{-1} did not reduce the J^{HOH}_{net} response to cholera toxin in ileal loops.

Loperamide is assumed to bind to opioid receptors with subsequent antagonism of the production or effects of second messenger compounds generated in response to secretogogues[6,7]. The data above appear to indicate that intraluminal or other routes of supply of reported chloride channel antagonists did not reduce increments in $J^{Cl}_{s->m}$ induced by cholera toxin or theophylline, while loperamide seemed to be partially effective in reducing chloride channel activity in vivo. For mechanistic purposes it is of some interest to examine the direct effects of loperamide on chloride movement through the apical conductance channel. Final concentrations of 2.5 mmol·l^{-1} loperamide, α-PC, DPC and NPEB all reduced the rate of conductive uptake of ^{36}Cl uptake by ileal brush border vesicles (Table 6). However the loperamide concentrations which were effectively antisecretory in vivo in the intestinal lumen were 20 and 100 µmol·l^{-1}. Lower concentrations of loperamide caused a slight enhancement of Cl uptake rates by the ileal brush border vesicle system (Table 6). Thus we have no evidence to support a direct

effect of normal pharmacologic doses of loperamide on chloride transport via the apical conductive pathway.

Discussion

The major criterion for judging the success of any antidiarrheal agent should be the reversal of net fluid accumulation in the intestinal lumen. On this basis the chloride channel antagonists tested in this study appear to hold little promise as antisecretory agents. The strategy employed has been based on the premise that a selective blocking of conductive chloride channels would be required to reduce the net fluid secretion caused by theophylline or cholera toxin. Selectivity of blocking is stressed because agents such as SITS which are recognised antagonists of the chloride-bicarbonate anion exchange process would be expected to reduce chloride absorption from the gut lumen. This action could reduce chloride and fluid absorption, and reduce possible antisecretory activity. α-PC has been shown to be relatively specific as a blocker of the conductance channel[16], but α-PC effects on secretory fluid flux into ligated loops were not sufficient to give any indication of usefulness as an antidiarrheal. The lack of effect via intralumenal dosing raises some question about accessibility of the inhibitor to the brush border membrane protein. Glycocalyx and unstirred layers at the cell surface may be more effective barriers in situ than in a brushborder vesicle system. The intraluminal accessibility question received some support from reductions in $J^{Cl}_{s>m}$ which approached statistical significance for intravenous and intraperitoneal routes of α-PC treatment. Significant reductions in J^{Cl}_{net} in the case of the intraperitoneal route were achieved almost entirely by reductions in the unidirectional secretory flux $J^{Cl}_{s>m}$. These in situ observations support the specificity of α-PC for conductive Cl channels as

observed previously. Unfortunately the apparent gains in inhibitor accessibility to the chloride channel via intraperitoneal administration had no major effect on the net secretory fluid flux observed in these intestinal loops.

The potent chloride channel blocker, NPEB, has been shown to be very effective in in vitro systems[17,18]. We hypothesized that the reported high affinity of this compound for the conductive channel might give significant reductions in $J^{Cl}_{s>m}$, even if the non-specificity of NPEB would be expected to block Cl ion absorption, and prevent antisecretory activity. As with α-PC, there may be problems with NPEB access to Cl transport sites in situ. This possible lack of access of chloride channel blockers to the ligand binding site presents a major obstacle to the theory of reversing secretory diarrhea with channel blockers.

Loperamide may have some antisecretory potential in the pig, as net fluid secretion induced by theophylline was significantly reduced by intralumenal loperamide. Antisecretory actions appeared to be occurring via changes in unidirectional chloride flux, and both the reductions in secretory flux and the increases in absorptive flux would be expected if loperamide were antagonizing the production of second messenger by theophylline. It is of interest that the loperamide effect apparently involved the same type of reductions in conductive chloride secretion which were to be expected from the use of the chloride channel blockers. The additional response to loperamide which may have reduced the net fluid secretion was the tendency toward an increase in the absorptive unidirectional chloride flux. Perhaps direct antagonism of the secretory chloride flux may not be sufficient to reverse a secretory diarrhea. Opiate agonists like loperamide may deserve more testing as intestinal antisecretory agents in the pig.

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Table 1. Effect of chloride conductance inhibitors on net jejunal fluid secretion induced by cholera toxin.

Condition	Trial # 1	Trial # 2
	Net fluid flux	
	$\mu l \cdot loop^{-1}\ min^{-1}$	
control	-16±16	-12±8
Cholera toxin	88±12	43±4
Cholera toxin + α-PC 0.2 mM	94±14	-
Cholera toxin + α-PC 2.0 mM	88±12	60±4
Cholera toxin + DPC 2.0 mM	-	38±4
Cholera toxin + NPEB 2.0 mM	-	36±3

n = 11 ± sem

Table 2. Effect of conductance inhibitors on basal net ileal fluid flux.

Inhibitor concentration	Net fluid flux			
$mol \cdot l^{-1}$	$\mu l \cdot loop^{-1}\ min^{-1}$			
	DPC	SITS	NPEB	α-PC
0	-65±15	-61±4	-68±23	-52±9
$1\ 10^{-6}$	-64±20	-60±9	-71±11	-62±6
$1\ 10^{-4}$	-74±13	-66±11	-77±10	-59±5
$1\ 10^{-2}$	-78±20	-44±9	-18±12	-40±5

n = 6 ± sem

Table 3. Effect of route of α-phenylcinnamate administration on theophylline - induced ileal fluid and chloride secretion.

Route of α-PC Supply	Parameter	Control		Theophylline	
		- α-PC	+ α-PC	- α-PC	+ α-PC
Intralumenal	J^{HOH}_{net}	-43±4	-	28±24	24±14
	$J^{Cl}_{s->m}$	5.8±1.6	-	8.0±0.5	7.5±0.7
Intravenous	J^{HOH}_{net}	-58±14	-47±6	83±16	64±7
	$J^{Cl}_{s->m}$	4.5±0.7	4.6±0.4	8.0±0.5	6.9±0.4
Intraperitoneal	J^{HOH}_{net}	-65±24	-74±5	22±14	13±12
	$J^{Cl}_{s->m}$	4.9±0.6	3.2±0.7	7.4±0.9	5.5±0.7
	$J^{Cl}_{m->s}$	5.6±0.3	6.6±0.4	3.7±0.5	4.0±0.5
	J^{Cl}_{net}	-2.9±1.0	-3.6±0.4	2.7±0.7	0.66±0.6

Units for J^{HOH}_{net} are $\mu l \cdot loop^{-1}\ min^{-1}$; for J^{Cl} the units are $\mu mol\ Cl \cdot loop^{-1}\ min^{-1}$

n = 12 ± sem

Table 4. Effect of 5-nitro-2-(2-phenylethylamino)benzoic acid on ileal fluid and chloride secretion induced by cholera toxin.

Condition	J^{HOH}_{net}	$J^{Cl}_{s\to m}$
	$\mu l \cdot min^{-1}$	$\mu mol \cdot min^{-1}$
Control	-13 ± 5	4.5 ± 0.2
Control + 2.0 mM NPEB	- 8 ± 8	4.9 ± 0.3
Cholera toxin	21 ± 6	5.2 ± 0.3
Cholera toxin + 2.0 mM NPEB	29 ± 9	5.9 ± 0.5

n = 15 ± sem

Table 5. Water and chloride flux after loperamide addition to ileal loops treated with cholera toxin.

Condition	$J^{H_2O}_{net}$	$J^{Cl}_{s\to m}$
	$\mu l \cdot min^{-1}$	$\mu mol \cdot min^{-1}$
Control	-17 ± 15	4.4 ± 0.3
Control + 20 µM Lop	-30 ± 9	4.6 ± 0.3
Control + 100 µM Lop	-29 ± 16	4.8 ± 0.5
Cholera toxin	15 ± 6	5.2 ± 0.6
Cholera toxin + 20 µM Lop	18 ± 6	4.7 ± 0.5
Cholera toxin + 100µM Lop	15 ± 8	4.1 ± 0.3

n = 10 ± sem

Table 6. Comparison of loperamide with chloride channel blockers for effects on conductive chloride uptake measured in ileal brush border membrane vesicles.

Condition	Experiment 1	Experiment 2
	Chloride uptake	
	nmol Cl / mg brush border vesicle protein / 10 sec	
Control	2.88 ± 0.12	2.28 ± 0.09
α-PC 2.0 mM	2.32 ± 0.07	-
DPC 2.0 mM	1.93 ± 0.10	-
NPEB 2.0 mM	1.37 ± 0.12	-
Lop 0.1 mM	3.20 ± 0.13	2.52 ± 0.20
Lop 0.5 mM	-	2.67 ± 0.12
Lop 2.0 mM	1.00 ± 0.02	0.75 ± 0.05

n = 8 ± sem

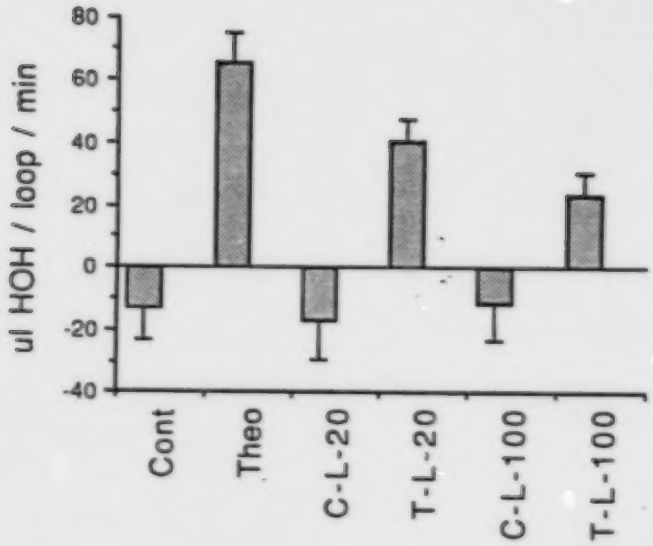

Fig 1 - Net fluid flux changes in ligated ileal loops after treatment with theophylline and loperamide. Effects of 20 and 100 µM loperamide addition to the electrolyte solution present in ileal loops in the control state, or containing 15 mM theophylline. C = distal loop fluid, T = distal loop fluid plus 15 mM theophylline. L-20 and L-100 refer to loperamide concentrations. Positive values represent net fluid secretion. n = 9 ± sem.

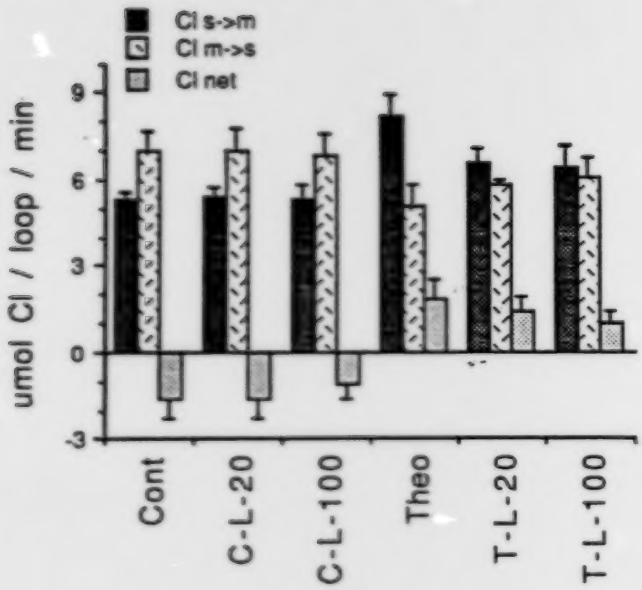

Fig 2 - Effects of loperamide on control and theophylline-induced chloride flux from the ileal lumen. Ileal loops loaded with fluid flux solution containing ^{36}Cl as a tracer and ^{3}H-PEG as volume marker were treated with 15 mM theophylline, and loperamide at 20 or 100 µM. C - L refers to control loops treated with loperamide, and T - L to loops receiving 15 mM theophylline and loperamide. Positive flux values represent ion movement into the intestinal lumen. n = 9 ± sem.

α-Phenylcinnamate inhibits chloride conductance in jejunal brush border membrane vesicles

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α-Phenylcinnamate has been characterized in terms of inhibitory effects toward the transport of chloride ion into pig jejunal brush border membrane vesicles. Buffer conditions and ion gradients were chosen to test the effects of α-phenylcinnamate on basal Cl transport, on conductive Cl transport, on anion exchange for HCO_3, and on self-exchange of Cl. Basal Cl transport involving a chemical gradient of Cl as the only driving force for uptake by vesicles was strongly inhibited by millimolar concentrations of diphenylamine-2-carboxylate (DPAC), 5-nitro-2(2-phenylethylamino)benzoate (NPEB), and to a lesser extent by 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS). Similar concentrations of α-phenylcinnamate did not reduce the basal rate of Cl uptake. Conductive Cl uptake driven by a potassium gradient was inhibited by ~50% at 2.5 mM α-phenylcinnamate. The inhibitor was equally effective in reducing the initial rate of conductive chloride accumulation in vesicles with naturally opened Cl channels (conductance activation by cyclic AMP and Ca^{2+}), or with Cl channels opened by exposure to tetramethylammonium buffer. Direct assay of the Cl-HCO_3 antiport activity in the vesicles indicated that there was no significant effect of α-phenylcinnamate on Cl-HCO_3 exchange at inhibitor concentrations which reduced conductance activity. Self-exchange rates of physiological concentrations of Cl were also unaffected by low mM concentrations of α-phenylcinnamate. Kinetic analysis indicated that α-phenylcinnamate was an uncompetitive inhibitor, requiring the presence of the normal Cl ligand for binding to, and inhibition of, conductive Cl transport by pig intestinal brush border vesicles.

Keywords: pig, chloride conductance, conductance inhibitors, intestinal secretion

Introduction:

Activation of Cl conductance in the jejunum is associated with fluid secretion and diarrheal disease. Potential therapeutic agents have been shown to enhance fluid absorption or block secretogogue release, but these agents tend to have undesireable side effects beyond the gastrointestinal system (4). The most specific target for antisecretory agents should be the membrane transport protein controlling the permeability of the apical membrane of the crypt cell to Cl ion. Several aromatic carboxylic acid derivatives such as anthracene-9-carboxylate, DPAC, p-(dipropylsulfamyl)benzoate, ethacrynate, and NPEB are reported to be inhibitors of Cl conductance activity in systems ranging from human tracheal epithelium to shark rectal gland tubule (1,3,8,11, 12,13,14).

A low toxicity, and the greatest possible specificity for the conductance protein would be desireable properties for Cl conductance inhibitors which could be used to treat intestinal hypersecretion. Anthracene-9-carboxylate is an effective conductance inhibitor in canine tracheal epithelium (12), but it is too toxic for clinical use. The band 3 anion exchange protein which is responsible for HCO_3:Cl antiport activity (7) is an essential component of the coupled absorption of Na and Cl from the intestinal lumen. The importance of maximizing ion and fluid absorption in a clinical disease state requires that potential conductance inhibitors should not affect anion exchange activity. DPAC is reported to have little inhibitory activity toward the band 3 protein of the thick ascending limb of the loop of Henle in rabbit kidney (3), but Reuss et al. (10) have reported substantial inhibition of the anion exchange activity in Necturus gallbladder epithelium by DPAC.

In screening several compounds including DPAC, NPEB and SITS for effects on Cl conductance activity we have observed that these inhibitors generally

tend to reduce the initial rates of Cl entry to pig jejunal brush border vesicles, both in the presence and in the absence of a transmembrane electrical gradient (Forsyth & Gabriel ms in preparation). When α-phenylcinnamate was tested in the same system the initial rate of Cl entry was reduced only when Cl entry was driven by a K^+ gradient. Basal rates of Cl transport were not affected. These observations stimulated the present investigation of the effects of α-phenylcinnamate on Cl transport in pig jejunal brush border vesicles.

METHODS

Vesicle preparation. Brush border membrane vesicles were prepared from pig jejunal mucosal scrapings as described previously (9). All steps in vesicle preparation were carried out at 0-4°. Scrapings from 40 cm jejunal segments were homogenized in 60 ml of buffer using four - 30 second bursts on a Brinkmann Polytron homogenizer. For in vitro activation of Cl conductance the homogenizing buffer was 300 mM mannitol, 1.0 mM $CaCl_2$, and 70 mM imidazolium-acetate at pH 7.4. Cell debris was removed by centrifuging crude homogenates for 10 min at 4500 x g. The supernatant was centrifuged for 60 min at 45000 x g to collect a crude vesicle preparation. These vesicles were resuspended in 30 ml of homogenizing buffer using one pass of a Potter-Elvehjem homogenizer at 1000 rpm. The suspended material was made 10 mM in Mg^{2+} by addition of Mg gluconate, and left for 20 min to allow aggregation of non-membranous material. The Mg^{2+}-aggregate was then removed by centrifuging for 15 min at 3000 x g. The activation system consisting of Na_2HAsO_4, 5.0 mM; KF, 5.0 mM; and ATP, 5.0 mM was incorporated by homogenizing (one pass of a Potter-Elvehjem homogenizer at 1000 rpm) with the vesicle suspension remaining after removal of the Mg^{2+} precipitate. The brush border fraction was collected

as a pellet by centrifuging at 27,000 x g for 30 min. Previous assay of marker enzyme activity has shown a 15-fold enrichment of brush border fractions, and minor amounts of contamination by basal-lateral material (6,9). Vesicles have also been prepared in 10 mM HEPES-tetramethylammonium (TMA) buffer. This procedure has been used to obtain vesicles which have Cl conductance activity with no requirement for addition of Ca^{2+} and cyclic AMP during the preparation procedure (5).

Measuring chloride uptake. Estimates of the initial rate of Cl uptake by the vesicle suspensions were obtained by measuring Cl uptake over a 10 sec interval. Cl uptake was stopped by diluting vesicle suspensions with ~500 volumes of isotonic NaCl. The ^{36}Cl content of the vesicle suspensions was determined by rapid filtration on cellulose acetate filters (0.45 μm pore size) followed by liquid scintillation counting.

Chloride conductance. Valinomycin (7.0 μg/mg vesicle protein) was added to vesicle suspensions 5 min prior to starting the uptake. For in vitro activation of conductance 20 μM dibutryl cAMP was also added at this time. Conductance conditions were established in vesicle suspensions by dilution with an equal volume of isotonic uptake media containing 100 mM K acetate and 1.0 mM KCl (1 μCi/ml of ^{36}Cl) as replacements for mannitol. For the basal condition K acetate (100mM) replaced an equiosmolar amount of mannitol in the buffer used to resuspend the vesicle pellet for Mg^{2+} precipitation, thus eliminating the K^+ gradient in the final uptake conditions. The chloride concentration was varied between 1, 11 and 51 mM by equimolar replacement of K acetate with KCl. When α-phenylcinnamate or other inhibitors were used, they were added to the vesicle suspension as a concentated stock solution in 70% ethanol at the same time as the valinomycin. The final ethanol concentration did not exceed 1.0% in the vesicle suspensions, and this concentration of

ethanol had no effect on the rate of Cl uptake by the vesicles. Conductance activity was expressed as the difference between the rates of Cl uptake measured in the presence and absence of the K^+ gradient.

Anion exchange. Anion exchange activity was measured in vesicles equilibrated with 100 mM mannitol, 50 mM HEPES-Tris (pH 7.5), and either 100 mM $KHCO_3$ or 100 mM K-gluconate. Uptake buffers contained 100 mM mannitol, 90 mM K-gluconate, 10 mM KCl (1 μCi ^{36}Cl/ml), and either 50 mM HEPES-Tris pH 7.5, or 50 mM N-morpholinoethanesulfonate (MES)-Tris pH 5.5. The activity of the anion exchanger was measured as the difference in initial rates of Cl uptake between vesicles prepared in gluconate buffer and mixed with uptake medium at pH 7.5 (no pH or bicarbonate gradient), and vesicles prepared in HCO_3 buffer at pH 7.5 with MES-buffered uptake media, pH 5.5 (pH and bicarbonate gradient).

Chloride self-exchange. Vesicles prepared with imidazolium-acetate buffer were used to study initial rates of chloride self-exchange. An equiosmolar amount of mannitol was replaced by 120 mM NaCl at the activation step following immediately after precipitation of non-brushborder material by Mg^{2+}. This step allowed equilibration of NaCl across the vesicles prior to measuring Cl exchange. The initial rate of Cl exchange was determined by diluting vesicles with a equal volume of the activation buffer containing ^{36}Cl (1.0 μCi/ml).

NPEB was synthesized as described by Wangemann et al. (11) and purified by thin layer chromatography and C:18 reverse phase HPLC. The identity of the synthetic product was verified by elemental analysis and nuclear magnetic resonance spectroscopy.

Kinetic constants were estimated by fitting the conductance rates to direct linear plots according to Cornish-Bowden (2) using Enzpac software obtained from Elsevier Biosoft. Inhibition type and affinity constants were derived from K_t and V_{max} values obtained for each inhibitor concentration, also using the Enzpac software.

RESULTS

There may be several transport processes mediating transmembranous chloride movement in intestinal brush border membrane vesicles. The major pathways for Cl uptake through the apical membrane occur via anion exchange, and conductive Cl transport. There may also be several minor transport pathways as well as a degree of leakiness associated with partial resealing of some brush border membrane fragments. The overall effect of these carriers is seen as a substantial rate of uptake of Cl into pig jejunal vesicles when the driving force is provided only by the chemical gradient for Cl. Measurements of inhibitor effects on the basal transport rate could give a useful indication of the specificity of potential inhibitors of Cl conductance, since this basal rate of Cl uptake may represent varying degrees of activity of any of the uptake modes suggested above.

The rate of Cl uptake under basal conditions was linear for the first 12 - 15 sec after Cl addition to the external medium (data not shown). This rate corresponds to a $t_{1/2}$ value of approximately 20 seconds for these vesicles, which equilibrate with a Cl space of 1.5 nmol per mg of vesicle protein (5,9). The initial rate of Cl uptake occurring in the presence of 11 mM extravesicular Cl ion is shown in Fig 1 for conditions of equilibrated intra- and extravesicular K^+. The basal rate of Cl uptake was reduced by 25% with 2.5 mM SITS. DPAC and NPEB were more inhibitory than SITS on this basal rate of Cl transport. In contrast to these reported inhibitors of anion exchange and Cl conductance, α-phenylcinnamate had no inhibitory effect when the driving force for Cl uptake was provided only by the Cl concentration gradient.

The lack of inhibitory actions of α-phenylcinnamate on basal Cl transport occurred with a range of Cl concentrations (Fig 2). At the three Cl concentrations used, the initial rates of Cl uptake in the absence of a K^+ gradient were not significantly altered by the presence of the conductance inhibitor. The slope components of the regression lines varied from + 0.22 nmol Cl $min^{-1} \cdot mg$ vesicle $protein^{-1}$ per mM unit of inhibitor concentration to - 0.54 with 51 mM Cl. This lack of effect of inhibitor on basal Cl uptake rates was unique to α-phenylcinnamate among the Cl transport inhibitors which we have tested.

Conductance activity is reported here as the increment in the rate of Cl uptake by vesicles following addition of extravesicular K^+ and valinomycin. This incremental rate, described as Cl conductance, is obtained by subtracting the basal uptake rates shown in Figs 1 & 2 from the total Cl transport rate measured under conductive conditions. The effect of inhibitor on the conductive component of the Cl uptake rate was measured in the same way, as the incremental rate of Cl transport, with equal concentrations of inhibitor added to both the basal and the conductive condition. The effects of α-phenylcinnamate on these Cl conductance rates measured in vesicles with in vitro conductance activation by Ca^{2+} and cyclic AMP is shown in Fig 3. The initial rate of Cl uptake was inhibited consistently by α-phenylcinnamate even with Cl concentrations as high as 50 mM.

We have preliminary information that conductance sensitivity to inhibitors such as DPAC and anthracene-9-carboxylate is minimal in vesicles which depend on preparation in TMA buffers for expression of conductance activity (5). α-Phenylcinnamate was also tested for inhibitory effects on conductance measured in vesicles prepared in TMA buffer. The inhibitory effects observed with vesicles prepared in TMA were quite similar to those seen when conductance was

activated in vitro by treatment with Ca^{2+} and cyclic AMP (Fig 4). α-Phenylcinnamate did not inhibit the basal Cl transport rates which were used in calculating the conductance values reported in Fig 4. The major differences between the vesicle preparations was the lower conductance velocity obtained with TMA-buffered vesicles (6).

Since the basal rate of Cl uptake occurring in the absence of the K^+ gradient may depend partially on the anion exchange activity it seemed desireable to directly assay the anion exchanger in these vesicles to ascertain sensitivity to α-phenylcinnamate. Knickelbein et al. have shown that the rabbit ileal brush border exchanger is dependent on HCO_3^- ion as well as a pH gradient for maximal activity (7). We have reported that similar assay conditions are required for the pig jejunal anion exchanger (5). Initial rates of Cl uptake by the anion exchanger were linear for 15 sec (data not shown). Initial rate measurements were carried out in the presence of a 25-fold range of α-phenylcinnamate concentrations (Table 1). There was no reduction of anion exchange activity at the low inhibitor concentrations, but a slight reduction was present with 2.5 mM α-phenylcinnamate.

Cl concentrations in the lumen of the jejunum are normally somewhat higher than 50 mM. This raises some questions about the physiological relevance of experimental conductance inhibition obtained with 50 mM Cl concentrations. However the conductance system is subject to limitations of the K^+ gradient which can be used to generate the potential difference for driving the conductive uptake of Cl. We attempted to avoid this problem by replacing the conductance conditions with a Cl self-exchange system. Cl self-exchange was used to measure effects of α-phenylcinnamate on Cl transfer at physiological concentrations of the ion. As shown in Fig 5 there was essentially no effect of α-phenylcinnamate on the activity of Cl self-exchange.

The potential use of conductance inhibitors to intervene in secretory diarrheal disease raises the problem of inhibitor utility in the lumen of the small intestine in the presence of Cl concentrations in the 100 mM range. The type of inhibition of Cl conductance exerted by α-phenylcinnamate is an important determinant of potential inhibitor effectiveness. Kinetic analysis of the conductive effects shown in Fig 3 indicates a K_t of 33 mM for Cl in the in vitro-activated conductance system. The analysis of the data with the direct linear plot method indicated decreasing K_t values and V_{max} values with increasing inhibitor concentration (Table 2). This unusual effect corresponds to uncompetitive inhibition, where the inhibitor is binding to the complex formed between Cl and the conductance transporter. The same type of inhibition was detected whether vesicles were activated by treatment with Ca^{2+} and cyclic AMP, or by preparation in TMA buffer. With uncompetitive inhibition there is no K_I parameter. Calulated estimates for the K_{IES} term were close to 1 mM. This value for K_{IES} indicates that the inhibitor may be effective at attainable intraluminal concentrations.

DISCUSSION

The membrane vesicle system is well suited to kinetic studies of inhibitors of Cl transport. The properties of the vesicle system correspond to the Cl conductance responsible for in situ fluid secretion. Conductance is activated by incorporation of ATP, Ca^{2+} and cyclic AMP into this vesicle system (6). The conductance rates in the activated vesicles are equivalent to values of approximately 80 $\mu A/cm^2$ of mucosal surface when a correction factor is applied to convert surface area of mucosal sheets to vesicle surface area (6). Uniform populations of vesicles can be used with varying concentrations of Cl-ligand and of inhibitor.

However along with the advantages provided by the vesicle system we have to deal with the heterogeneity of Cl transport processes present in this tissue. Experimental conditions can be optimized to favor conductive ion transfer, or anion exchange, but the contributions of other carriers to the measured Cl uptake can still be difficult to assess. This is especially true for the "basal" or unstimulated component of the Cl uptake.

Partial inhibition of the basal rate by SITS suggests that a part of the non-conductive Cl uptake in these vesicles may be occurring through the anion exchange system. It seems likely that NPEB and DPAC are not very specific inhibitors of Cl conductance, as they reduce basal rates of Cl uptake to a greater extent than SITS. DPAC also inhibited the anion exchanger in Necturus gall bladder (10), and reduced both the conductive and the "basal" components of Cl uptake in pig jejunal brush border vesicles (Forsyth & Gabriel, ms in preparation). Landry et al. have recently shown that ethacrynate, and some of its analogues are conductance inhibitors in membrane vesicles prepared from bovine renal cortex (8). However they also demonstrate major inhibition of basal Cl uptake by ethacrynate. In comparison with these results, and by the criteria of lack of effect on basal Cl transport rate, α-Phenylcinnamate may have unusual specificity for conductance inhibition.

α-Phenylcinnamate had a reasonable potency for the inhibition of conductive Cl uptake in the intestinal brush border vesicle system. Thus it may be compared to other reported inhibitors of conductance such as anthracene-9-carboxylate, probenecid, ethacrynate, DPAC, and NPEB. Of this group of potential conductance inhibitors, only ethacrynate, NPEB and its propyl derivative (8,11) are reported to have substantially higher affinity for the conductance protein than the values obtained for α-phenylcinnamate in this study. However NPEB and its derivative have been studied in the thick ascending limb of the loop of Henle, and direct comparisons are not possible without a side by side testing of these compounds in intestinal tissue.

We have suggested previously that the "basal" Cl uptake could represent unstimulated activity of other carriers, such as the Cl-HCO_3 exchange system. The lack of effect of α-phenylcinnamate on this "basal" uptake hints at specificity of the inhibitor toward the Cl conductance carrier. These suggestions are supported by the minor effects of α-phenylcinnamate on Cl uptake under optimal anion exchange conditions.

Self-exchange of Cl was used to measure transport rates at physiological Cl concentrations, with the assumption that a substantial proportion of that exchange would be occurring via a conductance carrier. The lack of inhibitor effects on the self-exchange process was unexpected. Since the α-phenylcinnamate did not inhibit self-exchange we may conclude that either it does not inhibit conductance at higher Cl concentrations, or else that self-exchange is mainly occurring via the anion exchange carrier. The latter possibility seems more likely since α-phenylcinnamate was an effective conductance inhibitor with 50 mM Cl ion.

There is a substantial difference in the rates of Cl conductance activity between vesicles prepared with tetramethylammonium to produce chloride conductance versus those which are treated with physiological conductance activators (5,6). In spite of the differences in the properties of these Cl transport systems, the inhibitory effects of α-phenylcinnamate are similar. In both cases there was no action of the inhibitor on "basal" Cl transport, and in both cases the inhibition had an uncompetitive mode of action.

Uncompetitive inhibition involves inhibitor binding to the carrier-ligand complex, to prevent ligand transfer. This type of binding is unusual in that there is a requirement for ligand to produce effective inhibitor binding, instead of a competition between inhibitor and ligand for binding to the carrier. This situation should be suited to obtaining inhibitor effects

at concentrations significantly below physiological levels of Cl ion. Simultaneous binding of ligand and inhibitor to the carrier could require two positively charged groups on the carrier, although it is not possible from our results to say if the inhibitor binding site is an alternate site for Cl binding and transport. A systematic trial of the effects of varying pH on inhibitor action could give more information about the requirements for ligand and inhibitor binding to carrier protein.

Specific inhibitors for the major types of Cl transporters would aid in attributing ion movement to specific carrier proteins in both the basal and the stimulated states of Cl uptake. High affinity inhibitors specific to the conductive transfer of Cl could be useful in the identification and characterization of that ion carrier (8). Relatively non-toxic inhibitors of Cl conductance might be useful antidiarrheal agents if they were effective at attainable intraluminal concentrations, and did not significantly reduce the absorptive component of the Cl flux. The specificity and the mode of inhibitory action of α-phenylcinnamate suggest that this compound should be tested for effects on chloride flux and intestinal fluid secretion in an in vivo model. These tests are in progress.

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Table 1. Activity of $Cl-HCO_3$ exchange in vesicles exposed to α-phenylcinnamate

α-phenylcinnamate mmole/l	Cl uptake rate ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) Gluconate pH_i 7.5/pH_o 7.5	Bicarbonate pH_i 7.5/pH_o 5.5	Difference
NONE	10.3 ± 0.48*	20.1 ± 0.65	9.8
0.1	9.9 ± 0.42	20.0 ± 0.59	10.1
0.5	9.9 ± 0.38	19.1 ± 0.53	9.2
2.5	10.1 ± 0.41	17.5 ± 0.72	7.4

* means of 10 measurements ± s.e.m.

Table 2. Kinetic constants for chloride uptake calculated by the direct linear method in vesicles treated with α-phenylcinnamate.

α-phenylcinnamate mmole/l	K_t mM	V_{max} nmol $Cl \cdot min^{-1} \cdot mg\ protein^{-1}$	K_{iss} mM
None	33	15.4	—
0.1	26	12.6	0.45
0.5	16	7.9	0.52
2.5	13	6.2	1.69

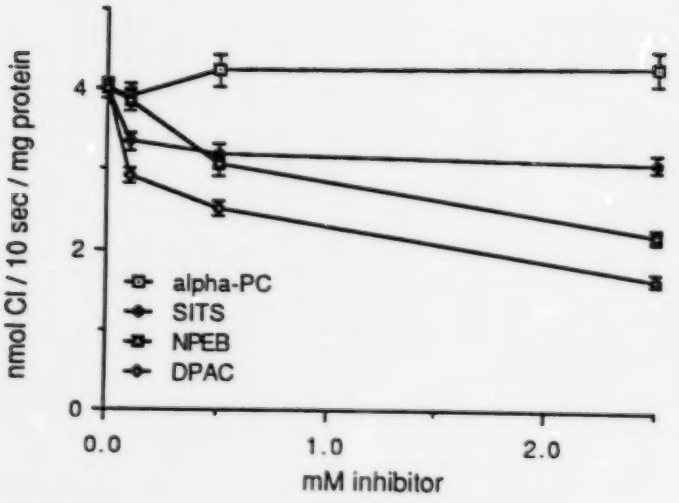

Fig 1. A comparison of the effects of Cl transport inhibitors on the initial rate of Cl uptake under basal conditions. Rates of Cl transport were measured in vesicle suspensions with equilibrated intra- and extravesicular K^+ levels. Cl uptake was initiated by adding 11 mM Cl (1.0 μCi ^{36}Cl per ml) to the suspension media. $n \geq 8 \pm$ standard error of the mean (S. E. M.). Missing error bars are smaller than the symbol size.

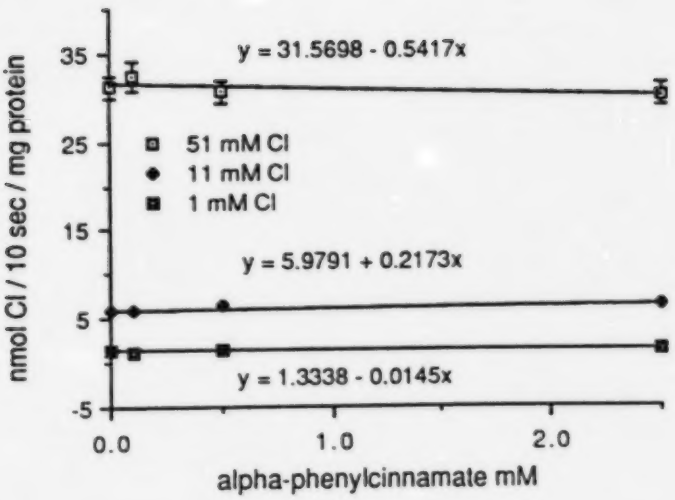

Fig 2. Effects of α-phenylcinnamate on the initial rate of Cl uptake under basal, non-conductive conditions. The effect of α-phenylcinnamate was measured at extravesicular Cl concentrations of 1, 11, and 51 mM. Dependence of initial rates of Cl uptake on α-phenylcinnamate concentration was calculated by linear regression. $n \geq 12 \pm$ S. E. M.

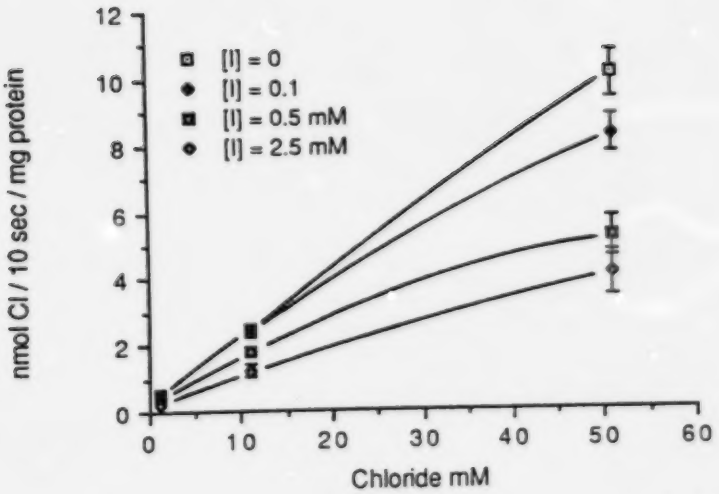

Fig 3. Effect of three concentrations of α-phenylcinnamate on the initial rates of conductive Cl uptake by membrane vesicles with in vitro activation of conductance. Cl conductance was activated in vitro by addition of Ca^{2+} and dibutyryl cyclic AMP. Conductance is shown as the means of the difference between the total rate of Cl uptake driven by a K^+ gradient, and the basal initial rate of Cl uptake measured without an imposed membrane potential. $n \geq 12 \pm$ S. E. M.

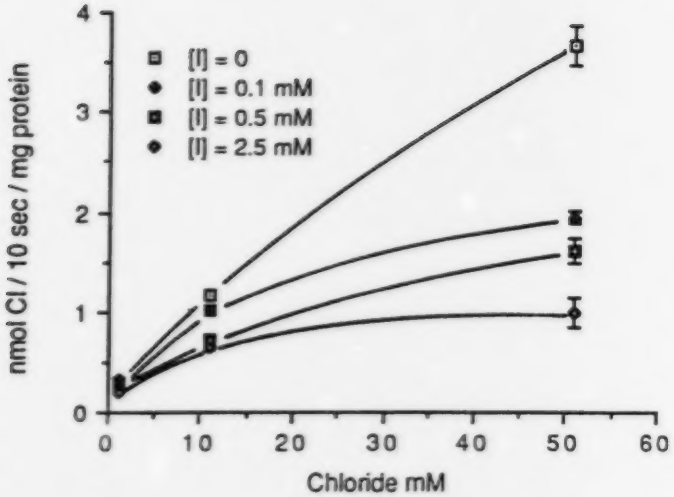

Fig 4. Effect of α-phenylcinnamate on the initial rates of conductive Cl uptake by membrane vesicles with conductance induced by tetramethylammonium. Conductance is shown as the means of the difference between the total rate of Cl uptake driven by a K^+ gradient, and the basal initial rate of Cl uptake measured without an imposed membrane potential. $n \geq 12 \pm$ S. E. M.

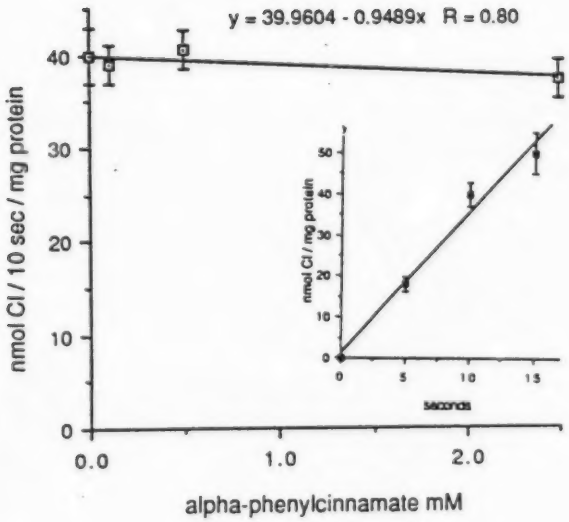

Fig 5. The effect of α-phenylcinnamate on the self-exchange of Cl across membrane vesicles. Self-exchange was measured in vesicles equilibrated with 120 mM Cl by adding ^{36}Cl (1.0 μCi per ml) to the suspension media. n = 8 $\pm$ S. E. M.. Initial rates of self-exchange are shown in the inset.